Preliminary In Vitro Evaluation of an Adjunctive Therapy for Extremity Wound Infection Reduction: Rapidly Resorbing Local Antibiotic Delivery

Stephanie R. Jackson,[1] Kelly C. Richelsoph,[1] Harry S. Courtney,[2] Joseph C. Wenke,[3] Joanna G. Branstetter,[3] Joel D. Bumgardner,[1] Warren O. Haggard[1]

[1]Joint Program in Biomedical Engineering, The University of Memphis and The University of Tennessee, Herff College of Engineering, 330 Engineering Technology Building, Memphis, Tennessee 38152-3210, [2]Veterans Affairs Medical Center and Department of Medicine, University of Tennessee, Memphis, Tennessee 38104, [3]U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas 78234

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ABSTRACT: Despite the continuing advances in treatment of open fractures and musculoskeletal wounds, infection remains a serious complication. Current treatments to prevent infection utilize surgical debridement and irrigation, and high doses of systemic antimicrobial therapy. The aim of this work was to evaluate, in vitro, the potential of a fast-resorbing calcium sulfate pellet loaded with an antibiotic. The pellet could be used as an adjunctive therapy at the time of debridement and irrigation to reduce bacterial wound contamination. Small pellets containing a binder and calcium sulfate were engineered to resorb rapidly (within 24 h) and deliver high local doses of antibiotic (amikacin, gentamicin, or vancomycin) to the wound site while minimizing systemic effects. Results from dissolution, elution, and biological activity tests against *P. aeruginosa* and *S. aureus* were used to compare the performance of antibiotic-loaded, rapidly resorbing calcium sulfate pellets to antibiotic-loaded crushed conventional calcium sulfate pellets. Antibiotic-loaded rapidly resorbing pellets dissolved in vitro in deionized water in 12–16 h and released therapeutic antibiotic levels in phosphate buffered saline that were above the minimal inhibitory concentration for *P. aeruginosa* and *S. aureus,* completely inhibiting the growth of these bacteria for the life of the pellet. Crushed conventional calcium sulfate pellets dissolved over 4–6 days, but the eluates only contained sufficient antibiotic to inhibit growth for the first 4 h. These data indicate that fast-resorbing pellets can release antibiotics rapidly and at therapeutic levels. Adjunctive therapy with fast-acting pellets is promising and warrants further in vivo studies. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:903–908, 2009

Keywords: drug delivery; calcium sulfate; infection; musculoskeletal trauma; bioresorbable

Infection remains a serious complication despite continuing advances in the treatment of complex musculoskeletal trauma. The emergence of bacteria resistance to traditional antibiotic therapies further complicates the treatment of these injuries.[1] Since U.S. military operations began in Afghanistan and Iraq, wound contamination with resistant bacteria has increased the occurrence of osteomyelitis and nosocomial infections in military hospitals.[1] To decrease the potential for wound infection, the current standard of care involves removal of all foreign material and necrotic tissue followed by irrigation and systemic antibiotic therapy.[2–4] High concentrations of systemic antibiotic are required to attain sufficient local levels in contaminated tissues with compromised vascularization. In the most difficult cases, multiple debridements and prolonged antibiotic therapy are required over several weeks to complete treatment.[4,5] Antibiotic toxicity, side effects, and evolution of bacterial resistance are problems associated with this course of therapy.[5,6]

Local delivery systems that place antibiotics directly at the contamination site are favorable alternatives or adjuncts because they minimize systemic toxicity and eliminate concerns about antibiotic penetration.[7–9] A local system capable of delivering antibiotics between debridement procedures could help reduce the incidence of infection in musculoskeletal trauma. Use of biodegradable drug delivery systems evades the need for additional surgery required by nonresorbable systems, such as polymethylmethacrylate (PMMA) beads.[6,10,11]

Calcium sulfate, a biocompatible, biodegradable material, is an interesting candidate for local delivery in cases of trauma because extensive bone damage is common. Calcium sulfate improves bone healing and repair, even when loaded with an antibiotic.[12,13] Calcium sulfate, optimized to resorb at a rate similar to that of new bone formation (several weeks to months) has been combined with antibiotics to eradicate existing bone infections in clinical and experimental situations.[2,5,7–9,13–16] A calcium sulfate pellet, engineered to dissolve and elute antibiotic over a shorter period of time, may be advantageous for infection prevention.

The concept of preventing infection by a more rapid dissolution of calcium sulfate and a faster antibiotic elution was investigated by Yarboro et al.[7] Conventional calcium sulfate pellets loaded with gentamicin (128 mg gent/g) and then crushed into flakes were applied to a *S. aureus* contaminated rat femur defect. They found no improvement in infection prevention over systemic antibiotic administration, but they found a significant improvement over systemic antibiotics with a local injection of gentamicin. They speculated that no improvement was observed over systemic gentamicin with calcium sulfate flake treatment because the sustained presence of calcium sulfate provided a substrate for bacterial adhesion. They also speculated that lower peak concentrations were seen with delivery from the flakes, which may explain why local gentamicin injection was superior to systemic antibiotics or calcium sulfate flakes.[7]

Correspondence to: Warren O. Haggard (T: 901-678-4346; F: 901-678-5281; E-mail: whaggrd1@memphis.edu)

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We hypothesized that a pellet can be loaded with an antibiotic, such as amikacin, gentamicin, or vancomycin, and made to dissolve and release local therapeutic levels of antibiotic within 24 h. Our long-range goal is to administer these rapidly resorbing pellets (FAST pellets) during initial debridement and irrigation, concurrently with systemic antibiotics, to reduce the level of bacterial contamination and the potential for infection in complex musculoskeletal injury. The use of calcium sulfate dihydrate and a binder gives these pellets their unique dissolution properties, since the pellets contain much larger and less tightly interconnected crystals than conventional pellets cast from alpha-hemihydrate.[17] The in vitro performance of the FAST pellet was also compared to crushed gentamicin-loaded conventional calcium sulfate pellets, similar to the concept employed by Yarboro et al.[7]

METHODS

Manufacture of Rapidly Resorbing Pellets

Antibiotic-loaded FAST pellets were made by mixing 10.0 g Food and Pharmaceutical grade calcium sulfate dihydrate powder (Terra Alba; US Gypsum, Chicago, IL) with 0.40 g sodium carboxymethylcellulose (CMC; Hercules, Wilmington, DE). A solution was prepared by mixing an antibiotic [0.42 g amikacin (amikacin sulfate; Bedford Labs., Bedford, OH), gentamicin (gentamicin sulfate; MP Biomedicals, Solon, OH), or 0.43 g vancomycin (vancomycin hydrochloride; Acros, Morris Plains, NJ] with 8.4 g deionized (DI) water. The solution was poured over the Terra Alba and CMC powders, and then the materials were mixed briskly for 1 min. The resulting 4% antibiotic-loaded paste was cast into silicone elastomer molds and allowed to dry for 24 h at room temperature. Once dry, the cylindrically shaped pellets (4.7 mm height, 3.4 mm diameter, 40 ± 2 mg weight) were demolded and sterilized using low-dose gamma irradiation (25 kGy) (Steris Isomedics, Libertyville, IL). Control pellets (calcium sulfate and CMC only) were cast using the same procedure. Pellets were characterized for calcium sulfate crystallinity by X-ray diffraction and differential scanning calorimetry, and overall morphology by SEM (Fig. 1).

Manufacture of Conventional Calcium Sulfate Pellets

Conventional calcium sulfate alpha-hemihydrate gentamicin pellets were cast by mixing 1.04 g gentamicin sulfate powder (gentamicin sulfate; MP Biomedicals) with 5.0 g DI water. The antibiotic solution was poured over 20.0 g calcium sulfate alpha-hemihydrate (calcium sulfate hemihydrate; Wright Medical Technology, Arlington, TN), and mixed briskly for 1 min.[9,14,18,19] The resulting 4% antibiotic-loaded paste was cast into silicone elastomer molds and left to dry overnight at room temperature. The pellets were then demolded and sterilized again using low-dose gamma irradiation (25 kGy). Each pellet was again 4.7 mm high, 3.4 mm diameter, and weighed 105 ± 4 mg. The pellets were crushed using a mortar and pestal into fine- and coarse-sized flakes; fine flake diameter was 150 μm (range, 0–300 μm), while coarse flake diameter was 600 μm (range, 0–1,200 μm). Flake size was verified using an inverting light microscope and image processing software (Bioquant Osteo II, Bioquant Image Analysis, Nashville, TN).

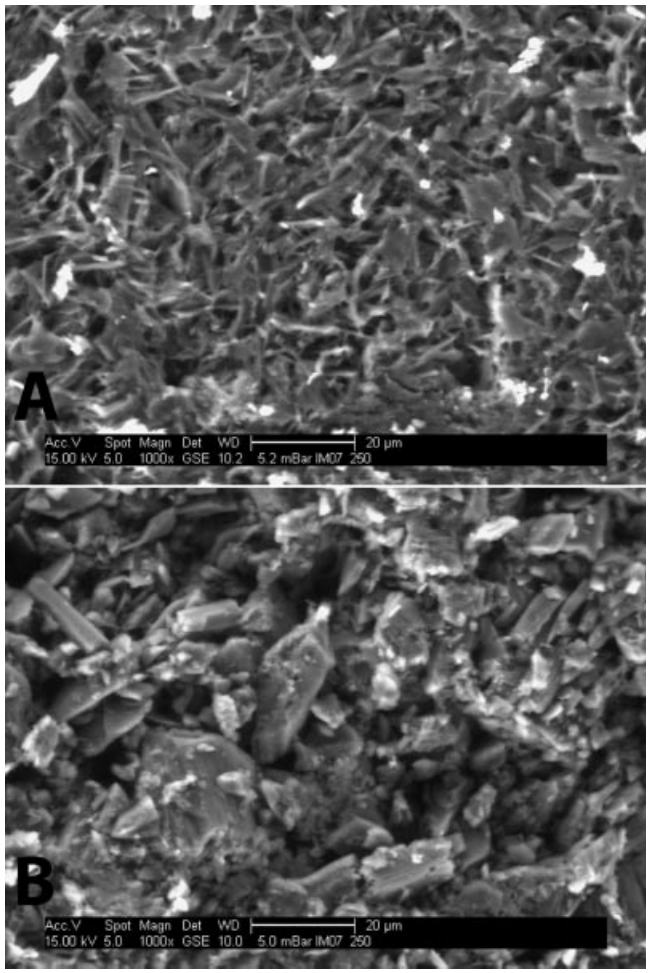

Figure 1. Environmental (Wet) Mode SEM images of (A) sterile conventional calcium sulfate pellet flakes or (B) FAST calcium sulfate pellets loaded with 4% gentamicin (original magnification, ×1000).

Dissolution Testing

To evaluate dissolution of the FAST pellets, individual pellets or the equivalent of one pellet in flakes ($n \geq 3$) were immersed in 100 ml of DI water in a 37°C water bath. At 1, 4, 8, 12, 24, 36, 48, 72, 96, 120, and 144 h, pellets were removed, oven dried at 37°C for 1 h, and weighed. The pellets were then reimmersed in fresh DI water. Testing continued until pellets were completely dissolved. Testing was repeated for three batches of pellets.

Elution Testing

Elution was characterized by placing groups of eight pellets or the flakes of eight pellets ($n \geq 3$) in 20 ml phosphate buffered saline (PBS) in a 37°C water bath. At 1, 4, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, and 240 h, aliquots were removed and frozen at −40°C. At each time point, the pellets were removed and placed in 20 ml fresh PBS. The amount of antibiotic in the eluates was determined (μg/ml) using a Florescence Polarization Immunoassay (TDxFLx; Abbott Laboratories, Abbott Park, IL). Testing was repeated for three batches of pellets.

Biological Activity Testing

Pseudomonas aeruginosa strain ATCC 27317 was used for biological activity testing of amikacin and gentamicin eluates.

A clinical isolate of *Staphylococcus aureus* Cowan I strain was used to assess activity of vancomycin eluates. The eluate having the lowest concentration for each antibiotic at each of the specified time points was used in the biological activity assay. *P. aeruginosa* and *S. aureus* were grown overnight at 37°C in trypticase soy broth (TSB). Conical tubes were prepared with 1.75 ml of TSB and 200 μl of antibiotic dilutions (amikacin: 0–640 μg/ml; gentamicin: 0–2,560 μg/ml; vancomycin: 0–160 μg/ml), 200 μl eluate samples, or 200 μl buffer. All tubes, except blanks, were inoculated with 50 μl of 1:50 dilution of bacteria. Blanks were supplanted with an additional 50 μl of TSB instead of bacteria. The tubes were vortexed and incubated at 37°C for 24 h. A blank was used to adjust the spectrophotometer to zero, and the absorbance at 530 nm (A_{530}) was recorded. Results were reported as percent growth relative to control, calculated as (A_{530} sample/A_{530} control) × 100. This method diluted the sample concentration by a factor of 10. Testing was repeated for three replicates of the samples.

Statistics

Statistical analyses included two-factor ANOVA and multiple comparisons tests with significance set at alpha = 0.05. For FAST pellets, differences in the dissolution or elution rate based on the antibiotic incorporated were evaluated. Gentamicin-loaded FAST pellets were compared to conventional pellet flakes by analyzing differences in dissolution or elution rate based on treatment type (FAST pellet, fine flakes, or coarse flakes).

RESULTS

Dissolution Testing

Sterile FAST pellets dissolved completely between 12–16 h (Fig. 2). Significant differences were only observed in the dissolution rates of gentamicin- and vancomycin-loaded pellets ($p < 0.01$), with gentamicin-loaded pellets dissolving more rapidly than vancomycin-loaded pellets. In general, for all antibiotics, 50%–70% of the pellet was dissolved within 4 h, 85%–90% within 8 h, more than 95% within 12 h, and no pellet remained after 16 h.

The dissolution rates were significantly different for fine and coarse conventional calcium sulfate pellet flakes ($p < 0.001$). Coarse- and fine-sized conventional gentamicin-loaded calcium sulfate pellet flakes dissolve in 4–6 days. Over 50% of the flake mass dissolved in 12 h for fine flakes and in 24 h for coarse flakes (Fig. 3).

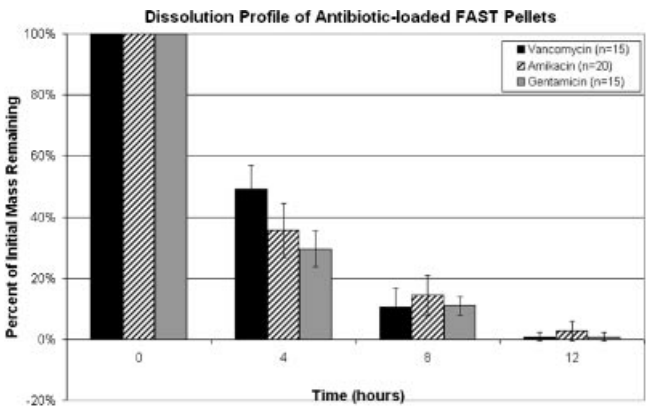

Figure 2. Dissolution of sterile FAST calcium sulfate pellets loaded with 4% amikacin, gentamicin, or vancomycin.

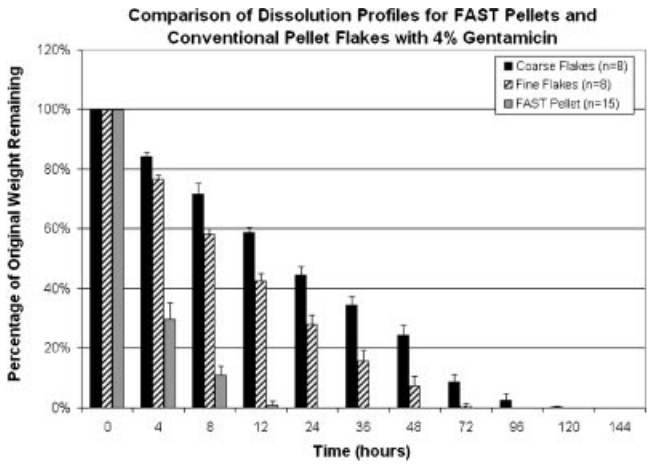

Figure 3. Dissolution profile comparison for gentamicin-loaded FAST pellets and gentamicin-loaded conventional calcium sulfate pellet flakes crushed into fine or coarse size.

Elution Testing

Differences were found in elution rates for vancomycin/amikacin ($p = 0.04$), but not for amikacin/gentamicin and vancomycin/amikacin (Fig. 4). Qualitatively, the elution profiles for gentamicin and amikacin both peaked at 1 h and decreased exponentially over the life of the pellets. The vancomycin elution profile showed a burst of antibiotic in the first hour, followed by a smaller release at hour 2, another burst at 4 and 8 h, and a relatively stable decrease for the remaining life of the pellet.

The elution rate for coarse and fine pellet flakes differed ($p < 0.001$) only over the first 4 h (Fig. 5). Similar elution profiles were found for coarse flakes and FAST gentamicin pellets for the first 24 h ($p = 0.740$). Elution beyond 24 h was not compared, since the FAST resorbing pellet sample had completely dissolved by this time point.

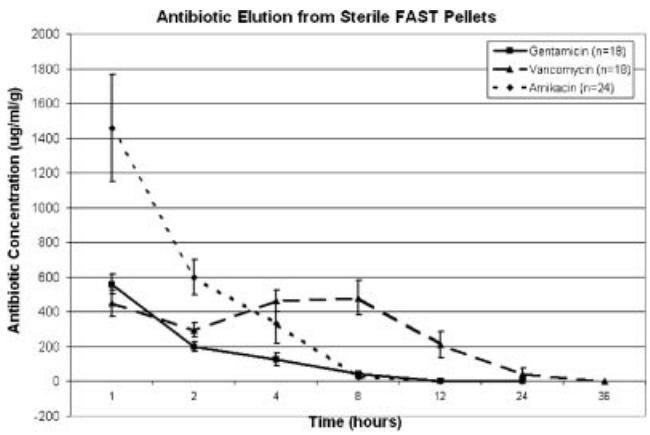

Figure 4. Elution of antibiotics from sterile FAST calcium sulfate pellets loaded with 4% amikacin, gentamicin, or vancomycin.

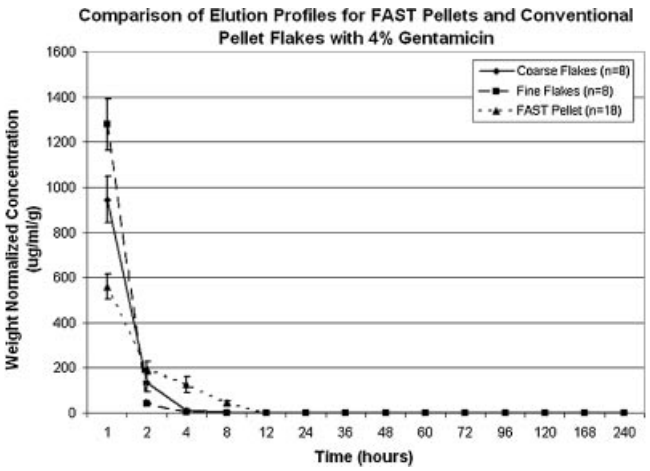

Figure 5. Elution profile comparison for gentamicin-loaded FAST pellets and gentamicin-loaded conventional calcium sulfate pellet flakes crushed to fine or coarse size.

Biological Activity Testing

The observed minimum inhibitory concentration (MIC) of *P. aeruginosa* ATCC 27317 treated with amikacin or gentamicin was 4.0 μg/ml (Table 1), within the range of the published literature.[20] Both gentamicin- and amikacin-loaded FAST pellet eluates completely inhibited growth of *P. aeruginosa* for 1, 2, and 4 h, as they maintained antibiotic concentration above the MIC. The MIC for *S. aureus* Cowan I clinical isolate with vancomycin was 0.5 μg/ml, within the range of the published literature.[21] Vancomycin-loaded FAST pellets were active against *S. aureus* through 12 h. At 24 h, the vancomycin concentration fell below the MIC. For both coarse- and fine-flake experiments, only the hour 1 sample contained sufficient antibiotic to inhibit growth of *P. aeruginosa*.

DISCUSSION

In recent years, calcium sulfate has been used extensively in local drug delivery, and has been effective in clinically treating chronic osteomyelitis when impregnated with tobramycin.[2,5,7–9,13–16,19] We evaluated the dissolution, elution, and biological activity of fast-degrading drug delivery vehicles made from cellulose binder and calcium sulfate dihydrate and from conventional calcium sulfate alpha-hemihydrate crushed into flakes. While this study used the Yarboro-type crushed conventional cast antibiotic-loaded calcium sulfate pellet as a comparison, the crushing/compressing of commercially available calcium sulfate pellets with tobramycin is not recommended by the manufacturer.[22] Our ultimate goal was to assess the potential of these two calcium sulfate-based carriers to reduce the incidence of infection in contaminated wounds by measuring their ability to release high concentrations of antibiotic shortly after administration in an in vitro environment.

Any new delivery vehicle should be versatile, since elution rate depends upon the interaction of carrier and antibiotic[23,24] and since emerging bacterial resistance is causing physicians to reconsider traditional antibiotic therapies.[20,21,25–28] We demonstrated that amikacin, gentamicin, or vancomycin can be delivered from the calcium sulfate dihydrate FAST pellets with effective dissolution and elution profiles. All FAST pellet formulations completely dissolved and eluted antibiotics in 12–24 h, whereas only 50% of the conventional pellet flakes had dissolved and 99.3% of antibiotic was released within this time frame. Variations in the crystal structure, and therefore, surface area, allows for the different dissolution profiles of these two delivery systems.[17,18,29]

The dissolution rate of the conventional calcium sulfate pellet was not substantially affected by the act of crushing, but its antibiotic elution was affected. The largest burst of antibiotic (1,278 μg/ml fine flakes with 96% of antibiotic loading or 945 μg/ml coarse flakes with 85% of antibiotic loading) occurred at 1 h, and then sub-MIC levels were released after 4 h for fine flakes and 8 h for coarse flakes for more than 15 days. Similar research

Table 1. Biological Activity Testing Results

	Organism				
	S. aureus Cowan I	*P. aeruginosa* ATCC 27317			
Timepoint (h)	RR+Vanc	RR+Amik	RR+Gent	CF+Gent	FF+Gent
1	−	−	−	−	−
2	−	−	−	+	+
4	−	−	−	+	+
8	−	+	+	+	+
12	−	+	+	+	+
24	+	+	+	+	+
48	+	+	+	+	+
240	/	/	/	+	+

RR, rapidly resorbing calcium sulfate pellet; Vanc, vancomycin; Amik, amikacin; Gent, gentamicin; CF, coarse conventional calcium sulfate pellet flakes; FF, fine conventional calcium sulfate pellet flakes; −, no growth; +, growth.

on the conventional, uncrushed pellets, which are designed to elute antibiotic over a period of 28 days and used clinically to eradicate an existing infection, release concentrations of roughly 1,000 μg/ml/g over the first 24 h and 10 μg/ml/g over the next 24 h, sufficient levels to prevent antibiotic growth for weeks.[9,14,18,28,29] With such immediate antibiotic elution from conventional flakes, the in vitro work of this study does not suggest that a large difference in initial antibiotic concentration would occur between flakes and an injection.[7]

Comparing the characteristics for the FAST pellet and the conventional flakes by Yarboro, we found that the FAST pellet dissolved more quickly and eluted antibiotic at a more sustained rate. This observation may pose a clinical advantage for administration of the FAST pellet in traumatic injuries. Our findings suggest that sufficient antibiotic concentrations are maintained to inhibit the growth of susceptible organisms for the life of the pellet. Just as conventional calcium sulfate pellets remain cohesive with up to 12% by weight of antibiotic loading,[29] the antibiotic loading of the FAST pellet could be adjusted to deliver higher antibiotics levels that may be required to target resistant organisms with MICs higher than those used in this study.

The limitations of our study arise from differences between in vitro and in vivo environments. In vitro data is an essential screening tool for new biomaterials. In measuring the drug release from a biomaterial, in vitro data can be used to compare different types of drug delivery systems. If one system was found to release more quickly than another, it would be expected that this difference would hold true in vivo, although the absolute rate of release may vary from an in vivo environment. Additional in vivo investigations are planned to determine if the in vitro findings of this study correlate to reduced risk of infection, biofilm formation, patient morbidity, and patient mortality in cases of complex musculoskeletal trauma.[7,30–36]

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